EXAMPLE 1

Construction of the BIBAC

[0044] The library features of the BIBAC are based on the bacterial artificial chromosome (BAC) cloning system described by Shizuya et al. (1992). In addition, the vector is a "state of the art" binary vector for *Agrobacterium*-mediated plant transformation (Hoekema et al. 1983).

[0045] Two major groups of components have been incorporated into the BIBAC. The first group includes those functions that are required in its bacterial hosts: *E. coli* and *A. tumefaciens*, and features to help characterize the library. The second group includes those features of the BIBAC which are intended to facilitate *Agrobacterium*-mediated plant transformation. A map of the BIBAC is shown in Figure 1.

[0046] The backbone of the BIBAC has the minimal region required for F factor replication and maintenance (O'Conner et al. 1989). The λ *cosN* and P1 *loxP* sites from pBAC108 are also incorporated into the BIBAC. These sites function as unique restriction sites to anchor one end of the insert and facilitate the analysis of partial digestions with restriction endonuclease(s). The *cosN* site can be cleaved with the bacteriophage λ terminase (Rackwitz et al. 1985); the *loxP* site by bacteriophage P1 Cre protein in the presence of the *loxP* oligonucleotide (Abremski et al. 1983). Restriction maps of the individual clones can be determined by indirect end-labeling and subsequent partial digestion (Rackwitz et al. 1985; Abremski et al. 1983; Kohara et al. 1987).

[0047] A marker which provides a positive selection for inserts is incorporated into the BIBAC. This marker is the *sacB* gene from *Bacillus amyloliquifaciens* (Tang et al. 1990) which encodes the protein levansucrase. Levansucrase was first identified as a 50 kD protein secreted

by *Bacillus subtilis* following induction by sucrose. The enzyme catalyzes transfructorylation from sucrose to various receptors (Dedonder 1966). The *sacB* structural gene of *B. subtilis* was cloned by Gay et al. (1983). Subsequently it was discovered that when 5% sucrose is present in agar media, the production of levansucrase is lethal to *E. coli*, *A. tumefaciens*, and *Rhizobium meliloti* (Gay et al. 1985).

[0048] The *sacB* gene was subcloned from the bacteriophage P1 cloning vector pAd10sacBII (Pierce et al. 1992). (The coding regions of the *B. amyloliquifaciens* and the *B. subtilis sacB* genes show 90% identity at the nucleotide level.) This construct has a BamHI cloning site region and a synthetic *E. coli* promoter upstream of the *sacB* structural gene. The BamHI cloning site is flanked by T7 and SP6 RNA polymerase promoters which can be used to generate RNA probes for chromosome walking. The *Bam* HI cloning site is unique to the BIBAC. When a DNA fragment is inserted into the *Bam* HI site, the *sacB* gene is inactivated, and the strain is viable when grown on media containing 5% sucrose. The *sacB* gene was introduced into pBAC108 to generate pCH1. This construct was electroporated into *E. coli* strain DH10B and the resulting strain was tested for sensitivity to high sucrose by plating a dilution series on standard LB media and on LB containing 5% sucrose. Cells plated on LB containing 5% sucrose showed a plating efficiency of less than 10^{-6} compared to cells plated on standard LB media. Thus, plating a potential library on 5% sucrose should yield primarily genomic clones.

[0049] The BIBAC has the replication origin region from the Ri plasmid of *A. rhizogenes*, the causal agent of hairy root disease. The Ti and Ri plasmids belong to different incompatibility groups and can be maintained stably together in one cell (White and Nester 1980; Constantino et al. 1980). This is important because many *A. tumefaciens* strains used for plant transformation

contain a disarmed Ti plasmid as the virulence helper plasmid. The minimal Ri origin region that is incorporated into the BIBAC has been shown to be sufficient for plasmid replication and stable maintenance at 1-2 copies per cell in *A. tumefaciens* (Jouanin et al. 1985). (The "2" copies per cell is included here to cover the presence of the duplicated DNA during cell replication.) The minimal Ri origin was subcloned from pLJbB11 which was characterized by Jouanin et al. (1985). The *oriT* cassette and the Ri origin were cloned adjacent to each other in pUC19 (Yanisch-Perron et al. 1985) to create pCH9. This was done so that the two components could be transferred to the vector as a single unit.

[0050] The BIBAC has an origin of conjugal transfer (*ori T*) derived from the wide host range plasmid RK2 of the Inc P group. When all other transfer functions are provided in *trans* by a helper plasmid, the *ori T* allows for the conjugal transfer of any covalently linked self replicating DNA (Ditta et al. 1980). The RK2 *oriT* of the BIBAC is subcloned from a DNA cassette in pNHKan-oriT constructed by Hengen and Iyer (1992). The BIBAC can be transferred to *A. tumefaciens* by conjugation. The RK2 *ori T* has been used to effect the conjugal transfer of the chromosomes of *E. coli* and *Rhizobium meliloti* (Yakobson and Guiney 1984). Therefore conjugal transfer of high molecular weight DNA from *E. coli* to Agrobacterium species and other bacterial species is possible. Alternatively, BIBAC clones can be introduced into *Agrobacterium* species and other bacterial species by electroporation. Mozo and Hooykaas (1991) reported that plasmids as large as 250 kb can be introduced into *A. tumefaciens* by electroporation.

[0051] Figure 2 illustrates the construction of the BIBAC. The right and left border sequences of the BIBAC are derived from the TL-DNA of the octopine plasmid pTiA6. The right border "overdrive" sequences are present (Peralta et al. 1986; van Haaren et al. 1987). The